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The Presence of an R467K Amino Acid Substitution and Loss of Allelic Variation Correlate with an Azole-Resistant Lanosterol 14α Demethylase in *Candida albicans*

THEODORE C. WHITE*

Department of Pathobiology, School of Public Health and Community Medicine, University of Washington, and Seattle Biomedical Research Institute, Seattle, Washington 98109

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Azole resistance in the pathogenic yeast *Candida albicans* is an emerging problem in the human immunodeficiency virus (HIV)-infected population. The target enzyme of the azole drugs is lanosterol 14α demethylase (Erg16p), a cytochrome P-450 enzyme in the biosynthetic pathway of ergosterol. Biochemical analysis demonstrates that Erg16p became less susceptible to fluconazole in isolate 13 in a series of isolates from an HIV-infected patient. PCR–single-strand conformation polymorphism (PCR-SSCP) analysis was used to scan for genomic alterations of *ERG16* in the isolates that would cause this change in the enzyme in isolate 13. Alterations near the 3′ end of the gene that were identified by PCR-SSCP were confirmed by DNA sequencing. A single amino acid substitution (R467K) that occurred in isolate 13 was identified in both alleles of *ERG16*. Allelic differences within the *ERG16* gene, in the *ERG16* promoter, and in the downstream *THR1* gene were eliminated in isolate 13. The loss of allelic variation in this region of the genome is most likely the result of mitotic recombination or gene conversion. The R467K mutation and loss of allelic variation that occur in isolate 13 are likely responsible for the azole-resistant enzyme activity seen in this and subsequent isolates. The description of R467K represents the first point mutation to be identified within *ERG16* of a clinical isolate of *C. albicans* that alters the fluconazole sensitivity of the enzyme.

Oral candidiasis is one of the earliest and most frequent opportunistic infections associated with immune system failure in human immunodeficiency virus (HIV)-infected individuals (8). Candidiasis is usually treated effectively with oral fluconazole or other azoles (28). Recently, the efficacy of these drugs has been compromised by the emergence of azole-resistant strains of *Candida albicans* and intrinsically resistant species such as *Candida glabrata* and *Candida krusei* (25, 29). One study estimated that >33% of isolates from patients with AIDS have MICs of fluconazole greater than 12.5 μg/ml, while standard susceptible strains have MICs of less than 4 μg/ml (18).

As the frequency of resistance has increased in the HIV-infected population, the mechanisms that result in azole resistance have become a focus of attention. Biochemical analysis of azole-resistant strains has documented changes in the target enzyme of the azoles, lanosterol 14α demethylase (Erg16p), and other enzymes in the ergosterol biosynthetic pathway (9). The azoles bind to the active site of Erg16p, a cytochrome P-450 enzyme, competing with the normal substrates of the enzyme (10). The *ERG16* gene has been cloned from *C. albicans* (14). Biochemical analysis has also documented decreases in the amount of drug that accumulates in the yeast cytoplasm (9). Recently, this reduced accumulation has been correlated with changes in efflux pumps that actively remove azoles from the cytoplasm (7, 21). Northern blot analyses have shown that genes from two families of efflux pumps (the *MDR1* and *CDR* gene families) are overexpressed in azole-resistant isolates (1, 31, 32). Genetic studies of both *Saccharomyces cerevisiae* and *C. albicans* have confirmed the importance of these efflux pumps in azole resistance (31, 32).

* Mailing address: Seattle Biomedical Research Institute, 4 Nickerson St., Suite 200, Seattle, WA 98109-1651. Phone: (206) 284-8846, ext. 344. Fax: (206) 284-0313. E-mail: tedwhite@u.washington.edu.

In 1994, Pfaller et al. (22) reported on an HIV-infected male who suffered from 14 episodes of oral candidiasis in 2 years who was treated with increasing doses of fluconazole. The MICs for a series of clinical isolates from the patient increased at intervals, starting with an MIC of 0.25 μg/ml and eventually rising to an MIC greater than 64 μg/ml. These increases correlated with the elevated doses of azole drug that were administered to the patient (27, 38). Several techniques were used to demonstrate that the isolates from this series are the same strain of *C. albicans* (22, 27, 38), although it was shown that a substrain was selected at isolate 2 (38). The resistance phenotype of the final isolate (isolate 17) is genetically stable, as it persists for over 600 generations in the absence of azoles. Recently, White (36) has shown that the overexpression of *MDR1*, *ERG16*, and *CDR* correlates with significant increases in resistance in the series (36).

The target enzyme of a drug is frequently altered as resistance develops in many biological systems (4). The purpose of the present study was to investigate the Erg16p enzyme in this series of 17 fluconazole-resistant *C. albicans* isolates by both biochemical and molecular techniques. Enzymatic assays were used to determine if the Erg16p enzyme activity is altered in this series. PCR–single-strand conformation polymorphism (PCR-SSCP) and DNA sequencing are used to recognize DNA sequences that have been mutated in the series. By using the 17 isolates, the timing of each genetic alteration within the resistance series was determined.

MATERIALS AND METHODS

Strains and growth of cultures. The *C. albicans* samples used in this study and the growth and storage of these cultures have been described previously (36, 38). As previously described, isolate 10 was not available for analysis.

Biochemical analysis. Inhibition of Erg16p enzymatic activity by fluconazole was evaluated as described previously (3, 20). Cell extracts were prepared as described previously (20). Briefly, cells were grown to mid- to late log phase in YEPD broth (36). Cultures were chilled on ice for 30 min, pelleted by centrif-

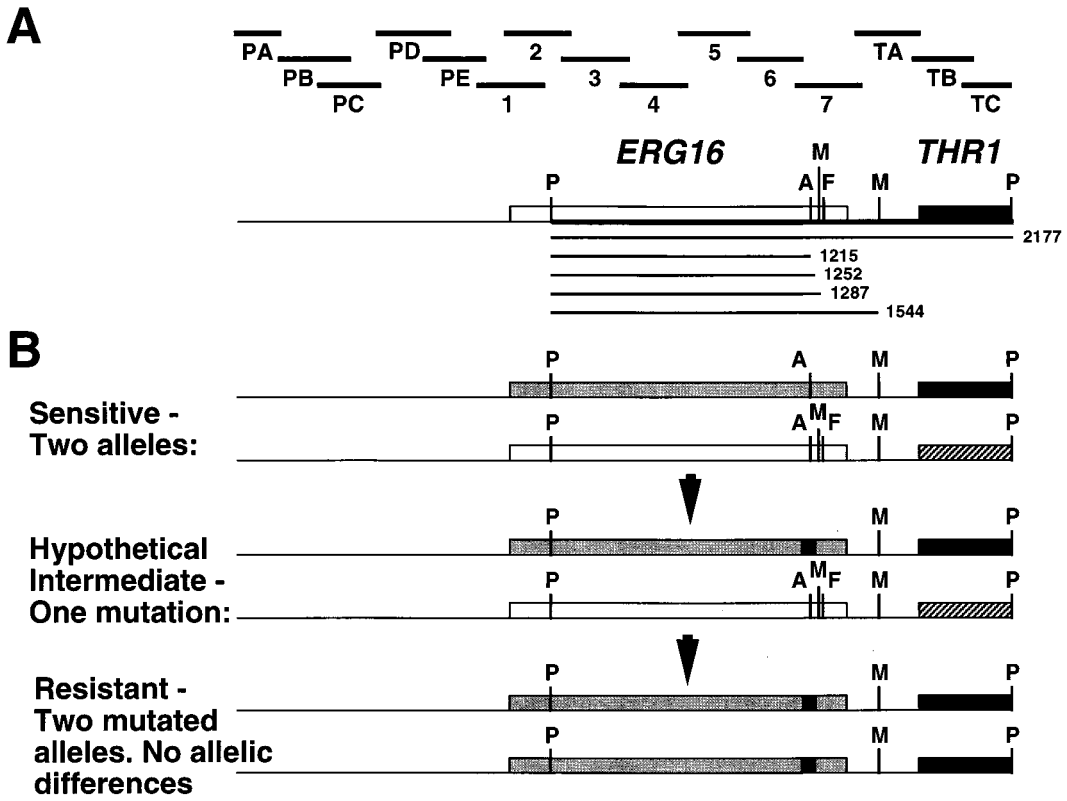

FIG. 1. (A) Genomic region surrounding the *ERG16* gene. The *ERG16* coding region (16) and the partial *THR1* coding region are diagrammed as shaded boxes. DNA fragments surrounding the *ERG16* gene (including regions of the *THR1* gene) that were used as probes and in PCR-SSCP analysis are shown above the line (see Materials and Methods for coordinates). Restriction enzymes important for the discussion are shown on the line, with abbreviations as follows: P, *Pst*I; A, *Acc*I; M, *Mun*I; and F, *Afl*II. DNA fragments important for the results are diagrammed below the line, including a *Pst*I fragment (2,177 bp), a *Pst*I/*Acc*I fragment (1,215 bp), a *Pst*I/*Mun*I fragment (1,252 bp), a *Pst*I/*Afl*II fragment (1,287 bp), and a *Pst*I/*Mun*I fragment (1,544 bp) that does not contain the first *Mun*I restriction enzyme site (at position 1252 from the *Pst*I site). (B) Diagram of loss of allelic variation of the *ERG16* gene region. Allelic differences were detected in the *ERG16* and the *THR1* gene sequences of isolate 1 (shown as different shadings in the top panel [labeled sensitive]). No allelic differences were detected in the two gene sequences in the resistant isolate (uniform shadings, bottom panel [labeled resistant]). Both alleles in the resistant isolate contain the R467K amino acid change (black box near the 3′ end of gene). The hypothetical intermediate (center panel) would contain the R467K amino acid change in only one of two alleles. Cells in which the loss of allelic variation occurred, selected for by an increased resistance to fluconazole as the result of the point mutation, would have the mutation in both alleles and coincidentally eliminate adjacent allelic differences.

ugation, and washed in phosphate buffer (0.1 M sodium phosphate, pH 7.5). Cells were resuspended in a small volume of phosphate buffer in a 50-ml conical centrifuge tube. Glass beads (0.5-mm diameter) were added to create a slurry, which was vortexed at high speed five times for 1 min each. Between each vortexing, the tube was cooled on ice for 1 min. Glass beads were removed by centrifuging the slurry through a disposable filter column (Fisher Scientific, Pittsburgh, Pa.) into a 15-ml conical centrifuge tube. The cell extracts were centrifuged for 10 min at 2,500 *g* and for 10 min at 10,000 × *g* to remove whole cells and cellular debris. The resulting extracts were frozen at −20°C until use. Protein concentrations were determined by a Bradford assay (Bio-Rad [Hercules, Calif.] Protein Assay) and typically ranged between 10 and 20 mg/ml.

Assays (1 ml) included cell extracts (1 to 10 mg of protein), 2 mM $MnCl_2$, 5 mM $MgCl_2$, 0.25 μCi of [^{14}C]mevalonic acid (25 Ci/ml; 70 mCi/mmol), and 50 μl of a cofactor mix. The cofactor mix contained NAD (14.2 mg/ml), NADP (16.6 mg/ml), NADPH (NADP reduced form) (19.2 mg/ml), glucose-6-phosphate (18.2 mg/ml), ATP (56 mg/ml), and reduced glutathione (18.4 mg/ml). The mix was made fresh and was neutralized to pH 7 with 10 M KOH (approximately 40 μl/ml) prior to use. Fluconazole was added to the assays when indicated. The reactions were prepared in glass test tubes and were incubated at 37°C for 2 h with shaking. One milliliter of 15% KOH in 90% ethanol was added to stop the reaction, a glass marble was used to cap the tube, and the reaction mixture was incubated for 45 min at 80°C. The reaction mixtures were cooled on ice and extracted twice with 3 ml of petroleum ether. The petroleum ether extractions were pooled and evaporated to dryness under a gentle stream of N_2. The residue was resuspended in 50 to 100 μl of petroleum ether and dotted on a thin-layer chromatography (TLC) plate (Silica Gel 60 without fluorescent indicator; EM Science, Gibbstown, N.J.). The plate was developed in toluene-ether (9:1, vol/vol). TLC plates were exposed to X-ray film or quantitated using a Storm PhosphorImager and ImageQuaNT software (Molecular Dynamics, Sunnyvale, Calif.). The labeled products that were detected by this analysis include several ergosterol biosynthetic intermediates from the following pathway: mevalonic acid → squalene → squalene epoxide → lanosterol → ergosterol. The target enzyme of the azoles, Erg16p, is part of the conversion from lanosterol to ergosterol.

PCR-SSCP analysis. Thirty oligonucleotides spanning the *ERG16* gene were used for PCR-SSCP and for sequencing. The coordinates of the two oligonucleotides that were used to produce each fragment are presented below. The order of the coordinates indicates the direction of the primer in the sequence, using the A of the start codon as position 1 (which corresponds to position 148 of the published sequence [16]). The fragments are diagrammed in Fig. 1A.

(i) Promoter region. This region contains fragments PA (position −1285 to −1268 plus position −1084 to −1100), PB (position −1100 to −1084 plus position −882 to −899), PC (position −899 to −882 plus position −629 to −645), PD (position −645 to −629 plus position −397 to −414), and PE (position −414 to −397 plus position −112 to −130).

(ii) Coding region. This region contains fragments 1 (position −147 to −130 plus position 153 to 136, 2 (position −15 to 3 plus position 276 to 256), 3 (position 256 to 276 plus position 554 to 535), 4 (position 531 to 548 plus position 828 to 811), 5 (position 801 to 818 plus position 1107 to 1087), 6 (position 1082 to 1100 plus position 1378 to 1360), and 7 (position 1348 to 1366 plus position 1653 to 1636).

(iii) Termination region. This region contains fragments TA (position 1630 to 1648 plus position 1933 to 1917), TB (position 1917 to 1933 plus position 2184 to 2163), and TC (position 2155 to 2172 plus position 2361 to 2343).

PCR-SSCP analysis was performed as follows. A 50-μl PCR mixture containing the following was prepared: 0.5 to 1.0 μg of genomic DNA, 40 pmol of each of two oligonucleotides, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0; 25°C], 0.1% Triton X-100), 2 mM $MgCl_2$, 100 μM each deoxynucleotide (dATP, dCTP, dGTP, and dTTP), 5 μCi of [α-^{32}P]dCTP (10 mCi/ml; 3,000 Ci/mmol), and 10 U of *Taq* polymerase. The PCR was performed under the following

conditions: 92°C for 3 min; 35 cycles of 92°C for 1 min, annealing temperature for 2 min, and 72°C for 3 min; 72°C for 10 min; and 4°C for storage. The annealing temperature (T_m) was determined empirically for each primer set, based on the following formula: T_m = 4 (number of G or C bases in the oligonucleotide) + 2 (number of A or T bases in the oligonucleotide). Most primer sets worked well at 50 or 55°C.

The PCRs were analyzed on 33-by-43-cm native polyacrylamide gels (10% glycerol, 1× TBE [0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA {pH 8.3}] 20% [29:1] acrylamide-bisacrylamide, 0.025% ammonium persulfate, 0.1% TEMED [*N*,*N*,*N*′,*N*′,-tetramethylenediamine]) in 1× TBE running buffer. A 3-μl aliquot of the PCR mixture was added to 9 μl of 0.1% sodium dodecyl sulfate–10 mM EDTA. A 3-μl aliquot of the dilution was added to 9 μl of stop buffer (80% formamide, 50 mM Tris-borate [pH 8.3], 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) and 2 μl of 0.1 M NaOH. These final dilutions were denatured at 95°C for 5 min and quickly cooled on ice. These (4- to 6-μl) samples were loaded on the gel, which was run at 15 to 25 W for 16 to 24 h. The gel was dried and exposed to X-ray film with an intensifying screen.

Sequencing. PCR mixtures were cleaned by adding 50 μl of 1× TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and 100 μl of phenol-chloroform-isoamyl alcohol (25:24:1). After vortexing, the samples were centrifuged at 14,000 × *g* for 5 min and the aqueous solution was loaded onto a spun column prepared as follows: 5 ml of Sephadex G-50-150 (Sigma) slurry in H_2O was loaded into disposable 5-ml filter columns (Fisher Scientific, Pittsburgh, Pa.), the column was placed in a 17-by-100-mm disposable tube and spun for 2.5 min at 2,000 × *g*. The flowthrough of the column was discarded, and a 1.5-ml Eppendorf centrifuge tube was placed beneath the column. The sample was layered on the top of the gel bed, and the column was respun. The flowthrough, containing the cleaned PCR fragment, was recovered in the Eppendorf centrifuge tube. The cleaned fragment was sequenced with its corresponding primers using the fmol DNA sequencing system (Promega, Madison, Wis.). Alternatively, PCR fragments were diluted to 2 ml with distilled water and spun through a Centricon 100 concentrator (Amicon, Beverly, Mass.) and washed with 2 ml of water and the sample was recovered per the manufacturer's instructions. These PCR fragments were sequenced with an ABI automated DNA sequencer with *Taq* dye-primer and dye-terminator chemistries (Applied Biosystems, Foster City, Calif.).

DNA manipulations: cloning and Southern blots. The promoter and termination regions of the *ERG16* gene were cloned by inverted PCR. Briefly, genomic DNA from strain SS was digested with *Bgl*II or *Pst*I and ligated at low DNA concentration (10 μg/ml). The DNA was purified by extraction with phenol-chloroform-isoamyl alcohol (25:24:1), extraction with chloroform-isoamyl alcohol (24:1), and ethanol precipitation before being used in PCRs. The promoter region was amplified by PCR using primers for position 153 to 136 and position 256 to 276. The termination region was amplified by PCR using primers for position 276 to 256 and position 1348 to 1366. After PCR amplification, the promoter region was cloned as a *Bgl*II/*Sac*I fragment into the *Bam*HI/*Sac*I sites of Bluescript II (SK−) (Stratagene, La Jolla, Calif.). The termination region was cloned as an *Acc*I/*Pst*I fragment into the *Acc*I/*Pst*I sites of Bluescript. Subsequent Southern blot analysis of genomic DNA indicated that the cloned segments accurately represented the genomic environment of *ERG16* (37).

DNA sequencing was performed with Sequetide (Amersham) and Sequenase (United States Biochemical) per Amersham's instructions. DNA preparation, DNA digestions, ligations, gel electrophoresis, transformations, Southern blotting, and random priming for radioactive probe preparation were performed according to standard published methods (2, 19, 30).

Nucleotide sequence accession numbers. The GenBank accession numbers are U67192 for the promoter region and U67193 for the terminator region.

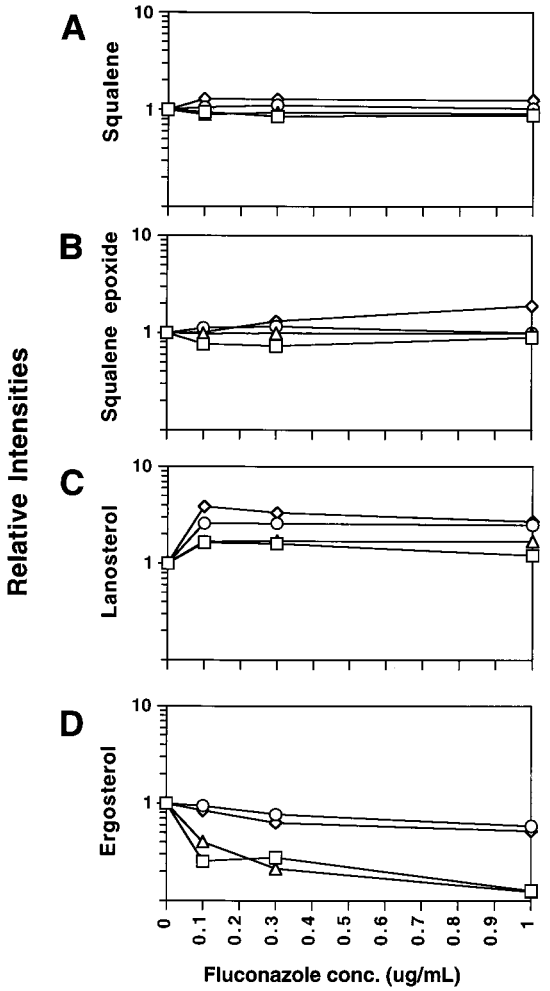

FIG. 2. Ergosterol biosynthetic intermediates formed in cell extracts by using [^{14}C]mevalonic acid as a precursor. Extracts were prepared from isolate 1 (□), isolate 12 (△), isolate 13 (○), and isolate 17 (◇). Reactions were run in the absence and presence of increasing doses of fluconazole (0, 0.1, 0.3, and 1.0 μg/ml [*x* axis]) and resolved on TLC plates. The amount of label was quantitated on a phosphorimager, and the values for each intermediate were graphed compared to the value obtained in the absence of fluconazole (relative intensities [*y* axis]). Intensities are graphed logarithmically to clearly show the differences in ergosterol levels between isolates. (A) squalene; (B) squalene epoxide; (C) lanosterol; (D) ergosterol.

RESULTS

Biochemical analysis of the Erg16p enzyme. The target enzyme of the azole drugs, Erg16p, was tested biochemically for indications that it became less susceptible to fluconazole at some point in the series of 17 isolates. The enzymatic assay (3, 20) used cell extracts and labeled mevalonic acid, which is an early precursor of ergosterol (see Materials and Methods), to produce labeled ergosterol as well as intermediates, including squalene, squalene epoxide, and lanosterol.

All extracts behaved identically in the absence of fluconazole, producing roughly equivalent amounts of ergosterol per milligram of protein (37). At increasing doses of fluconazole (0.1 to 1.0 μg/ml), differences between the isolates were observed (Fig. 2). Increasing concentrations of fluconazole did not affect the production of squalene or squalene epoxide (Fig. 2A and B). The concentrations of lanosterol were increased slightly, most likely as the result of the inhibition of Erg16p and the accumulation of precursors including lanosterol (Fig. 2C). The most significant changes are in the amount of ergosterol produced in the extracts (Fig. 2D). Ergosterol production in extracts from isolates 1 and 12 are decreased substantially with 0.1 μg of fluconazole per ml (50% inhibition at 0.06 to 0.08 μg/ml), while ergosterol production in extracts from isolates 13 and 17 remains active with fluconazole at concentrations of >1 μg/ml (50% inhibition at 1.0 to 1.12 μg/ml). This demonstrates that the enzyme in isolates 13 and 17 is at least 12-fold more resistant to fluconazole than the enzyme from isolates 1 and 12. No changes in enzyme susceptibility were observed in the series other than the change observed between isolates 12 and 13 (37).

Cloning of the promoter and termination regions of the *ERG16* gene. The regions 5′ and 3′ of the gene were cloned by inverted PCR (see Materials and Methods). The cloned promoter region extends for 1,285 nucleotides upstream of the start of the *ERG16* coding region. Several potential TATA boxes that might function as part of a promoter were present within 200 nucleotides upstream of the ATG. The promoter

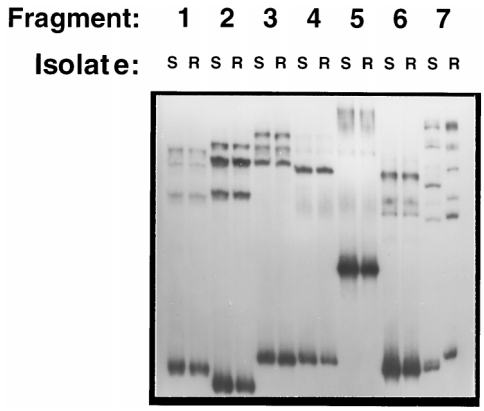

FIG. 3. SSCP analysis of DNA fragments 1 to 7. PCR fragments from isolate 1 (susceptible [S]) and from isolate 17 (resistant [R]) are shown.

region had no obvious homology to other sequences in the GenBank database.

The cloned region downstream of the *ERG16* gene was sequenced and found to contain an open reading frame with highly significant homology to the N-terminal third of the *THR1* gene that encodes homoserine kinase (ATP:L-homoserine-*O*-*P*-transferase), which catalyzes the first step of the threonine biosynthesis pathway. The 333-nucleotide noncoding region between the genes most likely includes the 3′ untranslated region of *ERG16* mRNA, the termination signals for the *ERG16* gene, and the promoter for *THR1*. There did not appear to be any TATA-like sequences upstream of the *THR1* start codon. The intergenic region did contain an unusual repeat sequence of $(AY)_{18}$, where Y represents pyrimidine.

PCR-SSCP of the *ERG16* gene. PCR-SSCP was used to identify specific base changes between isolates 1 and 17 in the *ERG16* gene region (Fig. 3). By comparing the SSCP patterns of DNA fragments from susceptible and resistant isolates, it was possible to identify DNA fragments containing genomic alterations. The DNA fragments used in the *ERG16* analysis (Fig. 1A) included five fragments from the promoter region (PA to PE), seven fragments from the coding region (1 to 7), and three fragments from the termination region that included the 5′ end of the *THR1* gene (TA to TC). Differences in the PCR-SSCP analysis of isolates 1 and 17 were seen with fragment 7 but not with fragments 1 to 6 (Fig. 3), all of which are within the coding region of the gene. This suggests that a sequence difference exists near the 3′ end of the coding region of *ERG16.*

In addition, PCR-SSCP differences were observed for fragments PA, PE, TA, and TC (37). These fragments represent untranslated regions of the *ERG16* gene that may be important in gene expression. SSCP differences seen in fragment TC suggested that sequence differences were present in coding regions of the *THR1* gene downstream of *ERG16.*

PCR-SSCP was used with DNA from all isolates in the series for each of the fragments that showed a difference in banding pattern (37). For all of the fragments, the change in SSCP pattern occurred between isolates 12 and 13.

Sequencing of the *ERG16* gene. The entire *ERG16* coding region was sequenced from isolate 1 and from isolate 17 by using PCR fragments. The initial and final isolates were selected for sequencing to identify any mutation in *ERG16* that occurred within the series. All of the differences that were identified by sequencing are located in the 3′ end of the gene in a region covered by fragment 7 (Table 1), consistent with the PCR-SSCP analysis. Four sequence differences were identified. A single nucleotide change occurred at position 1547; a G in isolate 1 was changed to an A in isolate 17. This transition resulted in an Arg-to-Lys amino acid substitution at position 467 in the Erg16p protein (denoted R467K). Two sequence positions were identified where a heterogeneity was identified in isolate 1, most likely the result of differences between the alleles of the *ERG16* gene in the diploid genome. Both heterogeneities were resolved in isolate 17 and neither of these two heterogeneities had any effect on the amino acid sequence. Finally, a single base change was identified in the 3′ untranslated region of the *ERG16* gene. Since the same nucleotide differs from the published sequence in both isolates 1 and 17, this difference is most likely the result of strain differences between the published sequence and the series under analysis.

TABLE 1. Differences in *ERG16* gene sequences

Position[a]	Nucleotide(s)			Amino acid		
	Published sequence	Isolate 1	Isolate 17	Published sequence	Isolate 1	Isolate 17
1547	G	G	A	Arg	Arg	Lys
1587	A	G and A	A	Leu	—[b]	—
1617	T	C and T	T	Asn	—	—
1767	T	A	A	Untranslated	—	—

[a] Positions (in nucleotides) are based on the published sequence (16). Standard numbering based on the A of the ATG start codon is lower by 146 nucleotides.

[b] —, no change from published sequence.

The only difference in the DNA sequence that had an effect on the amino acid sequence was the single amino acid substitution R467K. This amino acid change occurred in a conserved region of the protein (Fig. 4). This region of the Erg16p protein was highly conserved even between mammals and yeasts. Two residues surrounding R467K were noteworthy: the Cys three amino acids downstream of R467K that coordinates the fifth position of the iron atom in the heme (24) and the Phe four amino acids upstream of R467K that is important for interactions with the heme (23). Therefore, R467K was closely linked to amino acids that interact with the heme cofactor, an essential part of the active site of the Erg16p enzyme.

To confirm the sequence analysis of PCR fragments, restriction enzyme digestion of genomic DNA was used to monitor

Homo sapiens	**F** G A G **R** H R **C** I
***Rattus* sp.**	**F** G A G **R** H R **C** I
Ustilago maydis	**F** G A G **R** H R **C** I
Saccharomyces cerevisiae	**F** G G G **R** H R **C** I
Candida tropicalis	**F** G G G **R** H R **C** I
Candida glabrata	**F** G G G **R** H R **C T**
Candida krusei	**F** G G G **R** H R **C T**
Candida albicans	**F** G G G **R** H R **C** I
Resistant mutant	**F** G G G **K** H R **C** I

FIG. 4. Alignment of *ERG16* amino acid sequences from yeasts and mammals. The region surrounding the R467K amino acid substitution is presented for known *ERG16* sequences and the resistant mutant. Residues in boldface type include F463 (thought to be important in interaction with the heme [23]), R467K, C470 (which coordinates the fifth position of the iron atom [24]), and I471T (a position at which a Thr is found in the two intrinsically resistant species, *C. glabrata* and *C. krusei* [5]). The amino acid residues are translations of cloned genes. The sequence sources and GenBank reference numbers are as follows: *Homo sapiens*, D55653; *Rattus* sp., D55681; *Ustilago maydis*, Z48164; *S. cerevisiae*, M18109; *Candida tropicalis*, M23673; *C. glabrata*, S75389; *C. krusei* S75391; and *C. albicans*, X13296.

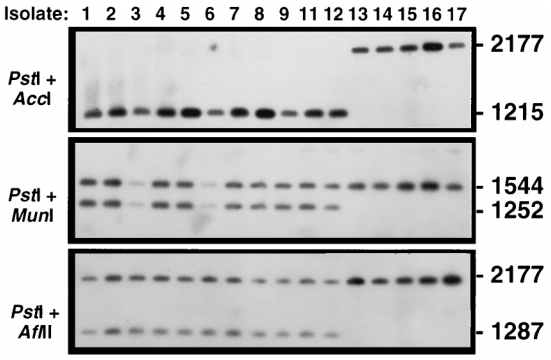

FIG. 5. Restriction enzyme analysis of loss of allelic variation in the series of isolates. Genomic DNA from each isolate in the series was digested with *Pst*I and *Acc*I, *Pst*I and *Mun*I, or *Pst*I and *Afl*II. The DNA was electrophoresed in an agarose gel and blotted onto nitrocellulose, and the filter was probed with fragment 6 (Fig. 1A). After hybridization at 60°C, the blot was washed and exposed to X-ray film. The fragment sizes (in base pairs) are indicated on the right of the blot.

specific nucleotides. Fortuitously, the R467K mutation destroyed an *Acc*I restriction enzyme recognition site. Similarly, restriction enzyme sites were present at the two heterogeneities, positions 1587 and 1617, such that only one of the two sequences at each position was digested with the enzyme (*Mun*I at position 1587 and *Afl*II at position 1617). Genomic DNA from the series was digested with *Pst*I and with *Acc*I, *Mun*I, or *Afl*II (Fig. 1A). Southern blots of these digests were probed with fragment 6. In each case, the restriction enzymes confirmed the sequence analysis and demonstrated that the change in pattern occurred between isolates 12 and 13 (Fig. 5). Thus, all changes associated with *ERG16* that have been identified in this series occurred between isolates 12 and 13.

Loss of allelic variation. In addition to the changes in the 3′ end of the coding region, PCR-SSCP analysis identified four other fragments (PA, PE, TA, and TC) that showed differences in pattern between isolate 12 and isolate 13 (37). Fragment PE was selected for further analysis since it is most likely to include regions of the promoter for the *ERG16* gene. The PCR fragment was sequenced from both directions. A portion of that sequence is shown in Fig. 6. Several heterogeneities were identified in DNA from isolate 1 (lines 1 and 2), while no differences were identified in DNA from isolate 17 (lines 3 and 4). These differences included many heterogeneities (allelic differences) and a single base deletion (position −284). In comparing the sequences of fragment PE from isolates 1 and 17, it was clear that the allelic differences that were present in isolate 1 were eliminated in isolate 17. It is interesting that the allelic differences were all resolved to a sequence (isolate 17 sequence) that was identical to that of the cloned promoter fragment, which was from a different strain of *C. albicans* (see Materials and Methods). Partial sequence analysis of fragments PA and TC showed similar losses of allelic variation (37).

DISCUSSION

In the present study, the target enzyme of the azole drugs (Erg16p) has been investigated by both biochemical and molecular techniques on a series of clinical isolates that developed azole resistance. The Erg16p enzymatic activity became more azole resistant, the Erg16p gene (*ERG16*) acquired an amino acid substitution, and allelic variation in the vicinity of the *ERG16* gene was lost. All of these changes occurred between isolates 12 and 13 in the series, such that isolates 13 to 17 exhibit all of these characteristics. The R467K mutation is the first point mutation to be described in a clinical isolate of *C. albicans* that modifies the enzyme activity of Erg16p. Its contribution to the development of resistance in this series is currently being evaluated.

Cell extracts clearly showed that there is a 12-fold-reduced susceptibility of the enzyme in isolate 13 compared to that of the enzyme from isolate 12. The equivalent amounts of squalene, squalene epoxide, and lanosterol produced in the extracts indicate that other enzymes in the pathway were not appreciably affected by fluconazole (Fig. 2). Since the extracts used in this assay were cell free and unlikely to contain intact membranes, the azole-resistant activity seen in extract 13 (Fig. 2) cannot be attributed to efflux pumps in cell membranes.

Sequencing of the *ERG16* gene from isolates 1 and 17 identified a single point mutation (G to A, a transition) that resulted in a single amino acid substitution (R467K). This amino acid substitution resides in an important region of the *ERG16* gene, between two residues known to be involved in interactions with the heme moiety in the active site of the enzyme. The conservative amino acid substitution (Arg to Lys) may have been sufficient to alter the azole-susceptibility of the active site, while not affecting the interactions of the enzyme with its normal cellular substrates. The R467K point mutation is unrelated to T315A, a mutation in *C. albicans ERG16* that alters enzyme susceptibility and was created by site-directed mutagenesis based on knowledge of the active site of the enzyme (17). R467K in *C. albicans* is also unrelated to the well-characterized point mutation D310G in *S. cerevisiae* SG_1 (11, 39). R467K is in a different region of the protein from D310G and T315A, although all of these mutations are in close proximity to the active site of the enzyme.

Allelic variation is observed for isolate 1 in all regions of the *ERG16* gene, including the putative promoter and terminator, and even in the adjacent *THR1* gene (Fig. 5 and 6; reference 37), as expected for a clinical isolate. These allelic variations are eliminated in isolate 13 in this series, indicating that a

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                .         .         .         .         .         .         .         .
-367  AACAAAACTATAATGAGATATTCATGATCTTATAAGTGGTAAACACAAGTATAGTAAAAACTTATAATGTGTGTAGAATTTGAT   -284

Isolate  1:

          ----------------------------------------------------------------------------------      1
          --T----------A------------------------C-----------------C-G--------A---T-GC-GA-A--X      2

Isolate  17:

          ----------------------------------------------------------------------------------      3
          ----------------------------------------------------------------------------------      4
```

FIG. 6. Sequences from fragment PE of isolates 1 and 17. The top line of the figure shows the sequence of the cloned promoter, approximately 136 nucleotides upstream of the published sequence. PCR fragments from genomic DNA of isolates 1 and 17 were sequenced in both directions and compared to the cloned fragment. Because of allelic differences, certain positions in the sequence of isolate 1 showed heterogeneity (lines 1 and 2). At positions of heterogeneity, one of the two residues matched the cloned sequence (line 1) (dashes indicate matches with the cloned sequence). A single deletion (X) was detected at position −284. No heterogeneities were observed in DNA from isolate 17 (lines 3 and 4).

major alteration occurred in this genomic region. The simplest explanation for this change is a mitotic recombination or gene conversion as diagrammed in Fig. 1B. In this model, the point mutation that causes the R467K substitution occurred in one of the two alleles of *ERG16*. This would most likely increase the azole resistance of that cell. A mitotic recombination or a gene conversion event would then create a cell with two copies of the point mutation in *ERG16*. The alteration would not be restricted to the R467K point mutation. Potentially, long regions of one allele could be transferred to the other allele, and this would account for the loss of genetic variation surrounding the *ERG16* gene. Constant azole drug pressure in the patient would not cause the alteration but would select for the outgrowth of cells in which the event occurred. This hypothesis is based on the supposition that a cell with two copies of *ERG16* containing R467K mutations is more resistant than a cell with only one copy of *ERG16*.

Mitotic recombination and gene conversion have been described previously for *C. albicans* (33–35) associated with protoplast fusions of two diploid cells, and subsequent UV-induced gene segregation. However, the current analysis is the first description of this type of event in a normal, nonirradiated diploid cell. From the limited data, it is impossible to predict which process was involved.

Several other possible, though unlikely, explanations for the loss of allelic variation can be suggested. A gene or chromosome deletion may have eliminated one copy of *ERG16*, or mating between two cells carrying one copy of the R467K allele could have occurred (both are highly unlikely). The possibility of a gene or chromosome deletion can be addressed using a genetic system with a dominant selectable marker such as IMP dehydrogenase as has recently been described (15).

The changes in the target enzyme that are detailed above are first detected in isolate 13 and persist in the five highly resistant isolates (13 to 17). Isolate 13 was obtained from the patient only 17 days after isolate 12 (26). It is unlikely that all of these changes occurred in a cell line that became the dominant strain in just 17 days. Unfortunately, only one isolate was stored from each patient sample, so it is impossible to determine if a subpopulation with these alterations was a significant fraction of cells at the time that isolate 12 was obtained. It is likely that the changes that have been identified accumulated over time in a subpopulation and that this subpopulation was not the dominant population until isolate 13.

The timing of all three changes is noteworthy. At the same time that the enzyme becomes more resistant to fluconazole, the R467K mutation appears in both alleles of the *ERG16* gene and differences between the alleles are eliminated. This strongly suggests a correlation between the azole-resistant enzyme activity and the R467K mutation. Proof that the R467K mutation causes resistance will depend on a genetic analysis of the mutation in a defined laboratory strain in which other factors can be carefully controlled.

In addition to the genetic alterations associated with *ERG16* described above, overexpression of the *ERG16* gene has been recently documented by Northern blot analysis (36). This fourfold increase in mRNA levels also occurs between isolates 12 and 13. The increase in mRNA levels is unlikely to account for the resistant enzyme activity for several reasons. First, the 4-fold increase in mRNA levels is unlikely to account for a 12-fold increase in enzyme resistance. Second, studies with *Saccharomyces* show only modest increases in resistance associated with increased expression (6, 13). Finally, preliminary biochemical data shows that the protein levels and enzyme activity levels in the cell are the same in both susceptible and resistant isolates (12).

Elegant molecular and genetic studies have shown that two types of efflux pumps are important in azole-resistant clinical isolates of *Candida* (1, 31, 32). Recent Northern blot analysis has shown that these two types of pump are overexpressed in this series but at points in the series different from those of the changes described above (36).

The R467K mutation and the loss of allelic variation described above clearly result in an azole-resistant enzyme. This azole-resistant enzyme is likely to contribute to the increased MICs that are observed for isolates 13 to 17. However, an exact correlation is not likely, since many other factors may contribute to the overall MICs. The overexpression of two types of efflux pumps and *ERG16* itself has been shown to correlate with the MICs in the series (36). It is unlikely that the effects of overexpression of an efflux pump and the development of an azole-resistant enzyme are simply additive for the MIC for the yeast. In addition, it is possible that other factors not yet identified also contribute to resistance in these isolates.

As identified in this study, the R467K substitution and the loss of allelic variation in the *ERG16* gene locus are the first molecular characterizations of naturally occurring point mutations in the *C. albicans* genome that are associated with azole resistance, and the R467K substitution is only the second mutation identified in the *ERG16* gene of *C. albicans* that alters enzyme susceptibility. It will be useful to identify other mutations in *ERG16* and in other genes that may be important in antifungal resistance. With an inventory of possible resistance genotypes, it will be possible to assess the overall importance of gene mutations and overexpression in the azole-resistant strains of *C. albicans* that are arising in the HIV-infected population.

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